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Award Number: DAMD17-02-1-0536

TITLE: Discovery of Cyclic Peptide Estrogens and Antiestrogens

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REPORT DATE: May 2004

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

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20040706 091

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY
(Leave blank)**2. REPORT DATE**
May 2004**3. REPORT TYPE AND DATES COVERED**

Annual Summary (22 Apr 2003 - 21 Apr 2004)

4. TITLE AND SUBTITLE

Discovery of Cyclic Peptide Estrogens and Antiestrogens

5. FUNDING NUMBERS

DAMD17-02-1-0536

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REPORT NUMBER****9. SPONSORING / MONITORING****AGENCY NAME(S) AND ADDRESS(ES)**U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012**10. SPONSORING / MONITORING
AGENCY REPORT NUMBER****11. SUPPLEMENTARY NOTES****12a. DISTRIBUTION / AVAILABILITY STATEMENT**

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE**13. ABSTRACT (Maximum 200 Words)**

The identification of proteins involved in the initiation of disease and the identification of small molecules that modulate these proteins are of great importance for the discovery of improved therapeutics. For example, compounds that potentiate estrogen receptor-mediated gene expression comprise a large class of currently employed drugs. These compounds can both treat breast cancer and provide hormone replacement therapy. Although initially beneficial, over time current clinically prescribed compounds can exhibit deleterious side-effects that include the development of drug resistance and an increased risk of breast cancer.

We initially hypothesized that a recently described genetic system termed split-intein mediated circular ligation of peptides and proteins (SICLOPPS) (PNAS, 1999, 96, 13638-13643) could enable the identification of small cyclic peptides that exhibit estrogenic and antiestrogenic activity in recombinant yeast systems. However, preliminary data suggests that SICLOPPS does not function or express well in yeast. Current efforts are directed at using related systems to investigate oncogenic protein tyrosine kinases and to identify proteins involved in estrogen-responsive pathways. These studies may identify new drug targets involved in the proliferation of breast cancer.

14. SUBJECT TERMSestrogens, antiestrogens, cyclic peptides, whole-cell assays,
protein-protein interactions, solid-phase peptide synthesis**15. NUMBER OF PAGES**

9

16. PRICE CODE**17. SECURITY CLASSIFICATION
OF REPORT**

Unclassified

**18. SECURITY CLASSIFICATION
OF THIS PAGE**

Unclassified

**19. SECURITY CLASSIFICATION
OF ABSTRACT**

Unclassified

20. LIMITATION OF ABSTRACT

Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Research Summary:

Protein-protein interactions are critical elements of cellular mechanisms of survival. Permutations in these otherwise highly controlled interactions can promote or prevent human disease. The identification of protein interactions involved in the initiation of disease and the identification of small molecules that potentiate these proteins are of great importance for the discovery of improved therapeutics. This proposal is directed at identifying methods to identify and potentiate critical protein interactions involved in the proliferation of breast cancer.

In our initial proposal we proposed to test the hypothesis that combinatorial chemical libraries of cyclic peptides expressed in yeast using the split-intein mediated circular ligation of peptides and proteins (SICLOPPS) genetic system¹ would yield potent, stable, and cell-permeable modulators of ER-mediated gene expression. These compounds were designed based on segetalin natural products, which are cyclic peptides that exhibit estrogenic activity in animals.²⁻⁴ Promising compounds would be evaluated in whole-cell assays in an attempt to identify novel compounds as potential chemotherapies for breast cancer or for hormone replacement. However, as described in the May 2003 research summary the construction of a functional SICLOPPS system in yeast proved more difficult than initially hypothesized due to low expression and no detectable function.

Although the SICLOPPS system failed in the preliminary stages, my graduate work has focused on investigating protein-protein interactions, in yeast genetic systems, that increase our understanding of the molecular basis of disease. One successful project under investigation involves the reconstitution of protein tyrosine kinase (PTK) initiated protein-protein interactions in a recombinant yeast system. PTKs catalyze the transfer of the gamma phosphate of ATP to specific tyrosine residues of target proteins to initiate protein-protein interactions that transmit signals critical for numerous cellular functions.^{5,6} More importantly, aberrant PTK activity has been implicated in numerous human disease states, including breast cancer.^{6,7} Through investigation of Abl and Src as model PTKs, I developed a yeast tribrid system employing a potentially universal PTK substrate that could be employed as a high-throughput proteomic tool to evaluate PTKs involved in disease.⁸ Furthermore, I modified this system to identify chemotherapeutically relevant compounds that inhibit PTK activity.⁹ This system could be further extended to investigate compounds with ability to inhibit PTKs specific to breast cancer proliferation. Current efforts, as outlined in my revised Statement of Work, are directed towards investigating this yeast tribrid system coupled with a recently designed fluorescence-based reporter gene towards screening of a human cDNA library to discover proteins with PTK activity. Discovery of novel PTKs could provide insight into mechanisms of breast cancer proliferation and provide new targets for the development of small molecule inhibitors as breast cancer therapy. This type of

screen could also be extrapolated to the discovery of previously uncharacterized proteins that have roles in the progression of breast cancer.

Conventional methods for screening cDNA libraries with yeast genetic systems can be time consuming pursuits that include the transformation of the cDNA library, growth for several days to generate colonies, combination and redistribution of colonies, and analysis of cDNA protein products for the desired activity. We envisioned that fluorescence activated cell sorting (FACS) might drastically streamline cDNA library screening in yeast genetic systems through the incorporation of a fluorescence based reporter gene. The power of flow cytometric techniques lies in the ability to quantify individual yeast cell characteristics based on fluorescence detection. The ability to evaluate single cells could potentially eliminate several steps in typical cDNA library analyses. Towards these ends, the yeast optimized green fluorescent protein¹⁰ (yEGFP) was incorporated into a reporter plasmid analogous to the conventional colorimetric-based (β -galactosidase) reporter plasmid pSH18-34.¹¹ Validation of this reporter plasmid to determine the sensitivity of the reporter and can be seen in Figure 1. As demonstrated in Figure 1, the yEGFP reporter provides excellent signal-to-noise by flow cytometric analysis and was deemed acceptable for incorporation into the yeast tribrid genetic system to identify PTKs from a cDNA library.

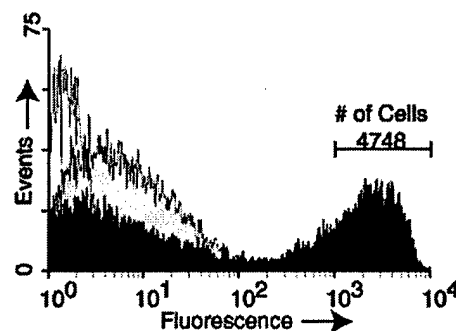


Figure 1: Validation of the yEGFP-based reporter gene. Overlaid histograms represent yeast transformants harboring no reporter (white), yEGFP reporter (light gray), and yEGFP reporter activated by a transcriptional activator (dark gray). The histogram gate illustrates the number of highly fluorescent cells resulting from the reporter activation.

The validation of the yEGFP reporter was critical for cDNA library screening pursuits utilizing FACS. We hypothesized that through the implementation of FACS, a "One-Tube" method for the combination of the conventional transformation, plating, and harvesting steps could be developed to greatly simplify cDNA screening. The One-Tube method simply involves the transformation of yeast with the DNA of interest (or cDNA library) and a specific genetic system, selection of desired transformants in liquid culture followed by induction of protein expression for fluorescent reporter gene expression upon appropriate protein-protein interactions, and FACS to isolate the most fluorescent yeast cells. After growth of sorted yeast cells, the gene of interest can be identified through standard molecular biological methods. Initial examination of the One-

Tube method with my previously established PTK yeast tribrid system coupled with flow cytometric analysis revealed the utility of these experiments for ability to isolate putative PTKs from a cDNA library (Figure 2). Identification of novel PTKs could provide insight into the progression of various human diseases, including breast cancer.

As seen in Figure 2, our current efforts have revealed that co-expression of a PTK along with the tribrid assay, incorporating a universal PTK substrate, results in quantifiable fluorescent reporter gene expression. Future work with this system (as stated in the statement of work) involves the evaluation of a human cDNA library in this yeast genetic system for the discovery of novel and the analysis of previously identified PTKs. These studies may provide new insight to the progression of breast cancers dependent on upregulated PTK activity.

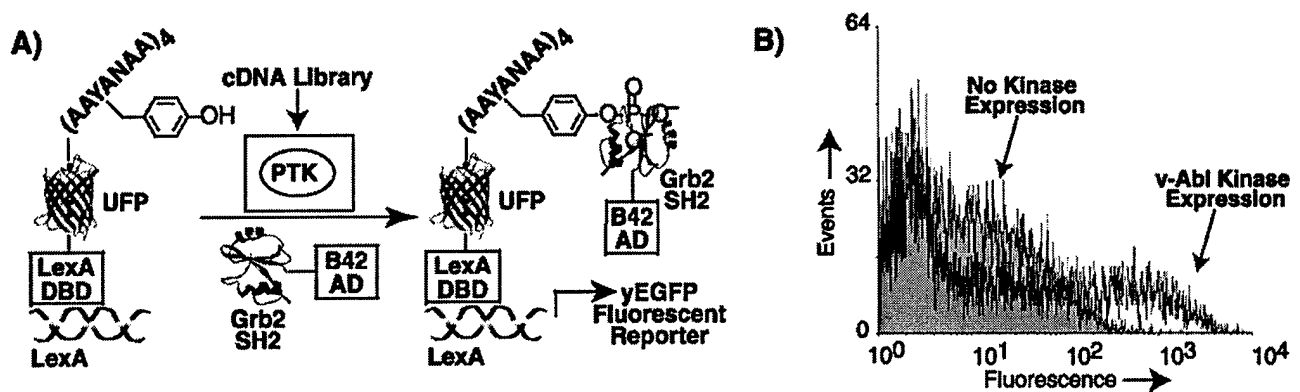


Figure 2: A) Schematic representation of the PTK-based tribrid yeast genetic system employed in these studies. Phosphorylation of the tyrosine residue by a kinase recruits the Grb2 phosphotyrosine binding protein to activate fluorescent reporter gene expression. B) Flow cytometry-based data for PTK (v-Abl) activated fluorescent reporter gene expression in the PTK-based tribrid genetic system illustrated in panel A.

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Key Research Accomplishments:

Development and validation of a yeast fluorescent reporter gene:

- Designed and evaluated a yeast fluorescent reporter gene that could be combined with flow cytometry to evaluate reporter gene expression.
- Incorporated and validated the fluorescent reporter gene for utility in PTK yeast tribrid systems for flow cytometric analyses

Development of a "One-Tube" protocol for the transformation and evaluation of cDNA libraries in PTK yeast tribrid systems:

- Adapted a previously reported PTK yeast tribrid system for increased utility in flow cytometry studies.
- Utilization of high-efficiency yeast transformation methods coupled to flow cytometry to evaluate protein-protein interaction in individual cells.

Initial evaluation of a human-derived cDNA library for putative PTKs:

- Employment of a novel fluorescent reporter gene, modified yeast tribrid system incorporating a universal PTK substrate, One-Tube transformation protocol, and fluorescence activated cell sorting (FACS) flow cytometry method to identify putative PTKs.

Reportable Outcomes:

Clark D. D., Peterson B. R. "Yeast Tribrid Systems: Covalent CIDs" Chem. Biol. *Manuscript in Preparation*.